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14. ABSTRACT

Purpose: The overall goal of this research is to preserve vision of patients recovering from severe facial burns by providing an improved method to reduce development of corneal defects, inflammation, infection and opacification. **Scope:** In this reporting period, amniotic membrane patches were modified by protein crosslinking to reduce their susceptibility to proteolytic digestion as a model for the observed degradation in inflamed eyes. An in vitro test system was used. **Major findings**: Five protein crosslinking methods differing in their molecular mechanisms were evaluated. A sensitive fluorescence assay was developed to measure enzymatic degradation of amnion on large numbers of samples. All five methods protected the amnion patches. Crosslinking decreased the rate of enzymatic degradation of amnion and decreased the total degradation after extended incubation with collagenase. The levels of protection varied from 35 to 100%. These results provide the basis for selecting the crosslinked amnion patches to be tested in an in vivo model of eye inflammation and for testing the effect of protein crosslinking on the pro-healing factors present in the membrane in Year 2.

15. SUBJECT TERMS
ectropion, cornea, burn scars, amniotic membrane, protein crosslinks, photomedicine, photochemistry, proteinases

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INTRODUCTION:

The overall goal of this research is to improve the visual outcomes as well as the quality of life for burn patients during the acute and convalescent phases of their rehabilitation. Scarring from second and third degree facial burns, and from subsequent skin grafts, causes the tissues involved to contract and, if significant enough, the patient is left unable to blink or close their eyes. This results in desiccation of the ocular surface, breakdown of the cornea's defense mechanisms and subsequent events that may lead to cornea opacification and the need for cornea transplant. Currently these patients receive frequent application of artificial tears, an imperfect solution that slows down their rehabilitation. Amniotic membrane (AM) transplantation to the ocular surface can assist in the maintenance of the ocular surface of these patients. However, commercially available membrane is not only very expensive, but enzymes on the inflamed ocular surface "dissolve" the AM very rapidly; in one day compared to two weeks in non-burn patients. In this project, our major goal is to stabilize the membrane by crosslinking it's constitutive proteins before applying it to the patient's eye. We will determine the crosslinking method that most effectively decreases the rate of enzymatic degradation of AM while preserving the beneficial anti-inflammatory and pro-healing factors in the membrane. In addition, we will evaluate photobonding as a sutureless, glueless alternative to sutures for attaching AM to cornea. We will also test an approach that combines AM with a hydrogel material to increase the ability of the amnion to hydrate the cornea. These studies will employ a rabbit model of eye inflammation.

BODY:

This Grant Agreement is a joint proposal with Col Anthony J. Johnson, MD, PI on Grant Agreement W81XWH-09-2-0069. The Statement of Work includes tasks to be carried at both the Massachusetts General Hospital and the Brooke Army Medical Center. An objective of the PRMRP was to enhance the translation of results from bench research to clinical problems and, in turn, to facilitate learning fundamental information from medical applications. To this end, Dr. Kochevar, and Dr. Johnson have communicated frequently by phone and Dr. Johnson visited Dr. Kochevar's lab in Boston in April (in connection with the World Cornea Conference.) Major tasks for Year 1 of this project were to evaluate protein crosslinking methods for stabilizing amniotic membrane (AM) against proteolytic degradation and to develop an animal model for eye inflammation to test the modified membranes.

> **Specific Aim 1.a. Determine the relationship between extent of protein crosslinking and the rate of enzymatic degradation of amniotic membrane in vitro.** Treat AM with photosensitization, UV radiation or transglutaminase to produce varying extents of protein crosslinking. Test resistance of modified membranes to proteolytic degradation.

Tissue can be stabilized against enzymatic degradation by covalent crosslinks formed between collagen chains using physical agents, such as light, and by chemical methods. To achieve the goals of this project, it is important to identify efficient crosslinking methods for AM proteins. Equally important is identifying which of these methods preserve the wound healing properties of AM while creating crosslinks. To achieve these combined goals, we selected crosslinking methods that operate by different molecular mechanisms and which involve different amino acid side chains in the covalent protein-protein crosslinks. In Year 1 our task for Specific Aim 1 was to address the former goal, namely, identify efficient crosslinking techniques. The effect of these techniques on healing properties of AM is a Year 2 task.

1.a.1. Preparation of amniotic membrane

Human placentas from scheduled caesarian section deliveries were obtained with the approval of the Institutional Review Board of the Partners Healthcare System. Placentas were washed with Earle's Balanced Salt Solution to remove residual blood clots. The AM was peeled away from the chorion and epithelial cells were removed by treatment with trypsin and scraping. Amnion was placed on nitrocellulose paper (stromal side away from paper) and cut into segments. The segments were stored at -80˚C in a 1:1 solution of 100% glycerol and Dulbecco's Modified Eagle's Medium with 1% penicillin-streptomycin. Immediately prior to use, membranes were defrosted and rinsed in distilled water for 45 min to remove all glycerol.

1.a.2. Enzymatic degradation of amniotic membrane.

Amniotic membrane was cut into ~0.4 cm x 0.4 cm pieces and weighed at room temperature and humidity. The weight varies with size and thickness but were in the 0.9-1.4 mg range. Three amnion samples were used for each treatment condition.

After treating AM with a crosslinking method, each sample was placed in separate 1 ml capped tube containing 1 ml of collagenase (Type I from Clostridium histolyticum; 0.10% w/v; 163 units/ml). The samples were then incubated at 37°C for the appropriate length of time (5 min to 20 hr). The untreated (positive) control AM (n=3) were not treated with crosslinking agent, but were treated with collagenase identically to treated samples. The negative control contained only collagenase (no amnion).

Collagenase (bacterial) hydrolyzes the amide linkage between glycine and X, when X is a neutral amino acid, in the (Gly-X-Y) repeating sequence that is characteristic of collagen. One amino group is produced at each cleavage site. Multiple enzymatic cleavages degrade the AM and release these peptide fragments into the medium. We measured the increase in peptide free amino groups in the medium to determine the extent of degradation of collagen in AM after various crosslinking treatments. The results were compared to the soluble peptide amino groups produced by collagenase proteolysis of untreated amnion (positive control).

1.a.3. Measurement of enzymatic degradation of amnion

Amniotic membrane is very thin (~50 µm) and, therefore, methods based on weight or swelling are not appropriate. The low amount of protein present in a small sample required a high sensitivity technique. We also needed a moderately high throughput assay in order to evaluate and optimize several crosslinking methods. Consequently, we adapted a fluorescence method that uses 96-well plates (1). In this assay, fluorescamine, a non-fluorescent reagent, reacts with free amino groups to form a fluorescent product. The fluorescence intensity is measured and used to calculate the percent degradation of the AM.

Protocol: Measurement of soluble free amino groups
The increase in free amino groups in soluble peptides was measured to determine the rate and extent of degradation of AM after various crosslinking treatments. For each sample, 10 µl of the supernatant containing soluble polypeptides (in triplicate) is added to 90 µl fluorescamine (Invitrogen Corp., 3.5 mM) in a well of a flat-bottomed 96 well plate and allowed to stand at room temperature for 5 min. The fluorescence was measured using a spectrofluorometer (Molecular Devices, SpectraMax M5) with 405 nm excitation and 470 nm emission wavelengths. The fluorescence intensity is converted into µM amine groups by comparison to a standard

curve prepared using glycine as the amine standard. The fluorescence in the negative control (no AM, only collagenase) is subtracted from all fluorescence measurements.

Percent protein degradation was calculated by dividing the amount of free amine groups in a sample by the free amine groups in positive control, then multiplying by 100.

1.a.3. Photochemical crosslinking

The energy of UV and visible radiation photons can be used to initiate chemical reactions that lead to formation of protein-protein crosslinks in collagen. The amount of UV and visible energy that is delivered to the surface of a target is called the Fluence with units of Joules/centimeter squared (J/cm^2). The rate that the energy is delivered is called the Irradiance with units of Watts/centimeter squared (W/cm^2). The fluence was varied by changing the exposure time using a constant irradiance, as shown by the relationship:

$$\text{Fluence } (J/cm^2) = \text{Irradiance } (W/cm^2) \times \text{Time (sec)}.$$

We evaluated two photo-crosslinking methods that utilize dyes (photosensitizers) to absorb visible radiation and initiate the crosslinking process; this is a relatively new method. In addition, ultraviolet (UV germicidal) radiation was used that is absorbed by collagen directly. These approaches were selected because their molecular crosslinking mechanisms differ and because they had been shown previously to crosslink collagen.

1.a.3.i. Riboflavin photosensitization

Riboflavin (vitamin B_2) is a micronutrient. A water-soluble derivative, riboflavin-5-phosphate (RF-5P), is a photosensitizer that initiates collagen crosslinking after it has absorbed UVA or blue light. RF-5P with UVA irradiation has recently been used to crosslink corneal collagen as a treatment for keratoconus (2). Thus, RF-5P appears to be a safe photosensitizer for modifying AM by crosslinking collagen. The mechanism for protein-protein crosslinking by RF-5P and blue light involves formation singlet oxygen, a reactive oxygen species that reacts with histidine and other oxidizable amino acid residues (3). Crosslink are believed to form from reaction of these oxidation products with the lysines on a different collagen chain (4).

For photocrosslinkng, RF-5P (1% in distilled water) is applied to the AM for 5 min and excess removed. The absorption spectrum of AM stained with RF-5P is shown in Figure 1. The RF-5P-stained AM was exposed to blue light (410-450 nm; 0.13 W/cm^2) from a Xenon lamp filtered for blue light (Focal Inc.) for varying lengths of time to deliver fluences up to 200 J/cm^2. RF-5P/blue light-treated AM samples and control untreated AM were incubated with collagenase for 20 hr at 37°C. Enzymatic degradation of AM was calculated from the amount of soluble amines released into the medium above the AM (see protocol above). The amount of amino groups formed by collagenase of untreated AM is taken as 100% degradation. The modified membranes showed lower amounts of degradation. The percent inhibition of AM degradation by RF-5P/blue light treatment is shown in Fig. 2A. A fluence of 100 J/cm^2 resulted in 45% inhibition of the degradation; higher fluences did not cause greater inhibition of degradation. The mean (± SD) % inhibition by RF-5P/blue light treatment was 35.5 (± 12) from 10 experiments (see summary in Table 1).

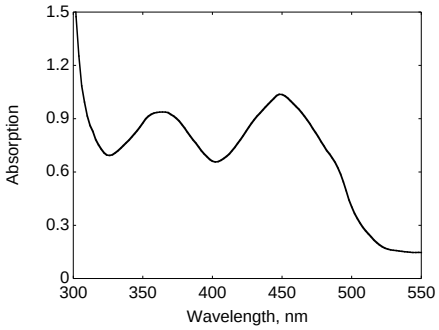

Figure 1. Absorption spectrum of amniotic membrane stained with 1% riboflavin-5-phosphate for 5 min.

The rates at which RF-5P-modified AM and untreated AM were enzymatically degraded was determined by incubating the membrane samples with collagenase for varying lengths of time before measuring the free amino groups in the supernatant. The RF-5P-stained AM were irradiated with 100 J/cm^2 blue light. As shown in Figure 2B, untreated control AM was enzymatically degraded more rapidly that the RF-5P/blue light-treated AM. A short delay (~20 min) was observed before degradation of the RF-5P/blue light-treated AM began. The initial slope (percent degradation/incubation time) after this delay was estimated. Comparison to the slope for the untreated control AM, the RF-5P/blue light-treatment decreased the rate of initial degradation by ~ 2-fold. In this experiment, the results obtained at long incubation times (22 hr) showed that RF-5P/blue light inhibited AM degradation by ~35% compared to the untreated control AM. ***These results indicate that RF-5P/blue light initiated modification of AM partially inhibits (~35%) the collagenase-mediated breakdown of the membrane.***

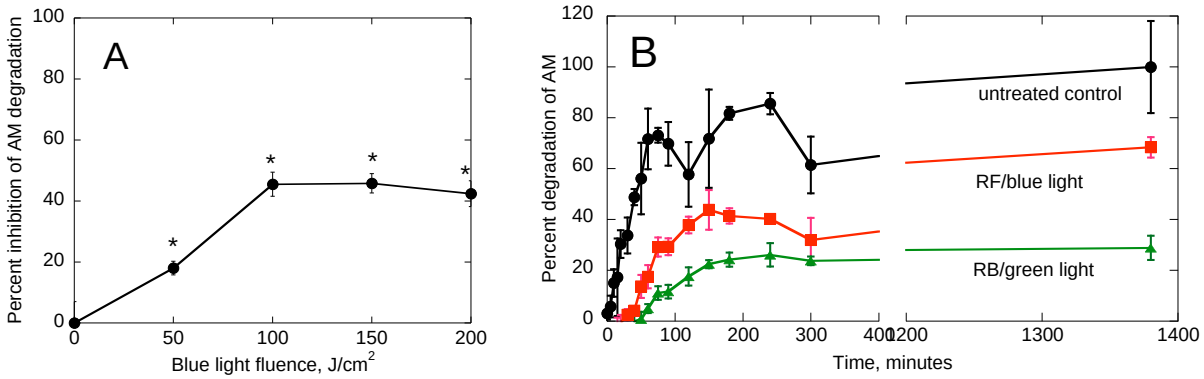

Figure 2. Photosensitized crosslinking of amniotic membrane. (A) Riboflavin/blue light treatment. Percent inhibition of collagenase digestion of RF-5P-stained AM after treatment with varying fluences of blue light. * $p < 0.05$ compared to untreated control AM. (B) Kinetics of collagenase-induced degradation of AM. Black circles = untreated AM. Red squares = AM treated RF-5P and 200 J/cm^2 blue light. Green triangles = AM treated with RB and 10 J/cm^2 green light.

1.a.3.ii. Rose Bengal photosensitization

Our previous studies indicated that treating tissues with Rose Bengal (RB) and green light initiated crosslinking of the tissue collagen (5-7). RB is FDA-allowed as a diagnostic for corneal abrasions suggesting that it will be acceptable as an amnion photocrosslinking agent. Basic studies have indicated that the mechanism for RB photosensitized crosslinking of proteins may differ in the presence and absence of oxygen. In the presence of oxygen, the RB excited triplet state can transfer energy to oxygen to form singlet oxygen, the same reactive oxygen species formed by RF-5P/blue light. The singlet oxygen then initiates protein-protein crosslinks as described above for RF-5P/blue light. In the absence of oxygen, RB excited states may undergo

electron transfer reactions with certain electron rich or electron poor amino acids in collagen (8) possibly leading to formation of radicals in the collagens and subsequent covalent crosslinks.

Application of 0.1% RB (in distilled water) to AM for 5 min produced an absorption of ~1 at 532 nm, the green wavelength used for excitation of RB. An absorption of 1 is equivalent to absorption of 90% of the incident green light (Figure 3A).

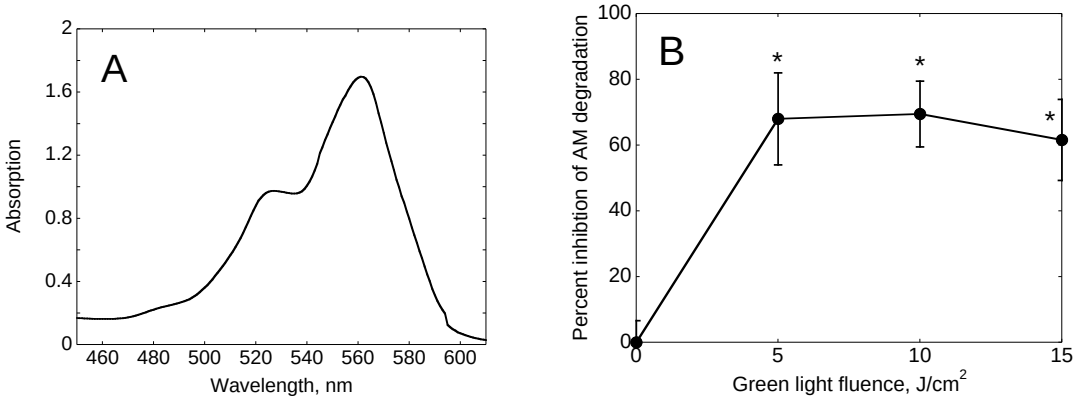

Figure 3. (A) Absorption spectrum of amniotic membrane stained with 0.1% RB for 5 min. (B) Percent inhibition of collagenase digestion of RB-stained AM after irradiation with varying fluences of green light. * $p < 0.05$ compared to untreated control AM.

For photo-crosslinking, RB-stained AM was exposed to 532 nm light from an OcuLight KTP laser (IRIDEX Corp, Mountain View CA). The irradiance was 0.13 W/cm^2. Light fluences up to 40 J/cm^2 were tested. The percent inhibition of collagenase digestion of the modified AM by RB/green light was calculated as described for RF-5P. An example of the results is shown in Figure 3B. RB-photosensitized collagen crosslinking inhibited collagenase digestion of AM by 65-70% percent using fluences of 5-15 J/cm^2. The mean percent inhibition (±SD) was 55 ± 13 in 10 experiments (Table 1).

The rate of amnion degradation after RB/green light treatment with collagenase was then determined. The amnion was treated with RB and 10 J/cm^2 green light, then incubated with collagenase for varying times. The results from one experiment are shown above in Figure 2B for comparison with RF-5P/blue light treatment. In this experiment, the maximum AM degradation of RB-modified AM was ~20% (i.e., 80% inhibition of degradation). The initial slope of the degradation verses incubation time curve, after a short delay, was ~25% of that for the untreated control amnion. ***These results indicate that RB/green light-initiated protein crosslinking modifies AM making it resistant (~55%) to enzymatic degradation.***

As mentioned above, based on previous photochemical studies the mechanism for RB photosensitized crosslinking may differ in the presence and absence of oxygen. The results presented above were obtained in an air atmosphere (~20% oxygen.) We directly compared RB/green light-initiated crosslinking of AM proteins (as detected by ability of collagenase to degrade AM) in 100% oxygen and in the absence of oxygen (nitrogen atmosphere). An atmosphere-controlled irradiation chamber was constructed for these studies; the differences in atmosphere in the chamber was verified by monitoring the photobleaching of RB on AM.

Three fluences were tested. The results are shown in Figure 4. In the presence of 100% oxygen, RB/green light treatment caused greater inhibition of AM degradation, i.e., greater amount of crosslinks, than the treatment in the absence of oxygen. This difference was greatest

for the lowest fluence, 5 J/cm^2. **This result supports the proposed difference in crosslink mechanisms in the presence and absence of oxygen and provides another alternative protein crosslinking method that may have less effect on the pro-healing factors in AM.**

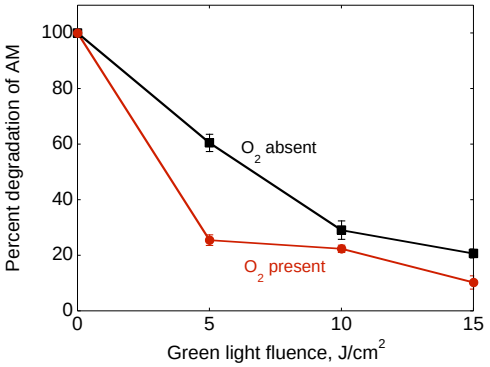

Figure 4. Comparison of Rose Bengal-photosensitized inhibition of enzymatic digestion of AM in the presence (red circles) and absence (black squares) of oxygen.

1.a.3.iii. UVC irradiation

Ultraviolet radiation has been used to crosslink tissue proteins, but has been associated with breakdown of protein chains in some cases. The proteins in amnion, especially the aromatic amino acids and histidine, directly absorb the 254 nm emitted by germicidal (UVC) lamps. Consequently, a photosensitizing dye is not required. Several molecular species are produced in the proteins including radical cations and free radicals. These species produce many types of protein-protein crosslinks by a variety of mechanisms.

A germicidal lamp was used for UVC irradiations (irradiance = 0.12 W/cm^2). Membranes were irradiated with fluences up to 120 J/cm^2 and, as shown in Figure 5A, the percent inhibition of degradation by collagenase increased with increasing fluence. A maximum level was not reached even at 120 J/cm^2. Higher fluences would require unrealistic treatment times; delivering 120 J/cm^2 required 2 hr 40 min. A fluence of 120 J/cm^2 inhibited of degradation by 48% (Figure 5A). In four experiments, the mean percent inhibition was 43 ± 6. AM treated with this fluence were then incubated with collagenase for varying times to determine the kinetics of enzymatic degradation. The results are shown in Figure 5B. The initial rate of AM degradation was ~2-fold lower for the membranes treated with UVC. ***These results indicate that UVC germicidal radiation partially inhibits (~43%) degradation of AM by collagenase.***

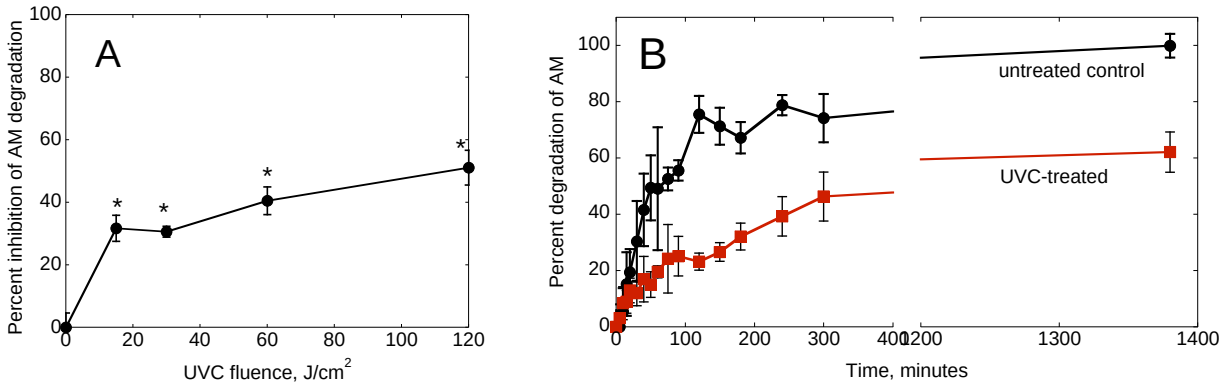

Figure 5. Photocrosslinking of amniotic membrane with UVC radiation. (A) Percent inhibition of collagenase digestion of AM by treatment with varying levels of UVC. * $p < 0.05$ compared to untreated control AM. (B) Rate of collagenase-induced degradation of AM treated with 120 J/cm^2 UVC (red squares) or of untreated AM (black circles).

1.a.4. Chemical crosslinking Many chemical agents have been used to crosslink structural proteins in a variety of tissues and in bioengineered tissues. We chose to evaluate two chemical agents that crosslink proteins by different mechanisms: glutaraldehyde, which bridges between amine groups on different chains, and a diimide, which facilitates linkages between protein carboxylic acids and amines.

1.a.4.i. Glutaraldehyde

Glutaraldehyde is a well known, effective and easily available crosslinking agent for structural proteins in tissues and has been used to crosslink proteins in AM (9). However, toxicity (possibly due to formation of formaldehyde by biodegradation) and calcification have been cited after its use as a crosslinking agent (10). Glutaraldehyde is bifunctional and can react with amine groups on two different protein chains to form Schiff bases (imines) links than can be reduced to secondary amines.

Amniotic membrane (0.9-1.3 mg) was treated with varying concentrations of glutaraldehyde up to 200 μM for 30 min in phosphate buffered saline, PBS. The AM was then thoroughly washed in PBS to remove excess reagents and byproducts. The membrane was cut and incubated with collagenase for 20 hr as described above. The level of soluble peptide amine groups present in the supernatant was analyzed and the percent inhibition of AM degradation calculated relative to control untreated AM. An example of the results is shown in Figure 6. Nearly 100% inhibition of degradation was achieved using 25 μM and higher glutaraldehyde. ***These results indicate that glutaraldehyde treatment can completely block degradation of AM by collagenese.***

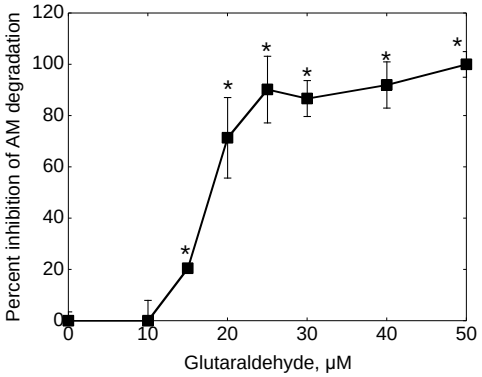

Figure 6. Crosslinking of amniotic membrane with glutaraldehyde. Percent inhibition of collagenase digestion of AM by treatment with varying concentrations of glutaraldehyde for 30 min. * $p < 0.05$ compared to untreated control AM.

1.a.4.ii. Carbodiimide

A carbodiimide was included in the agents being tested because a recently published paper described using this agent to enhance the physical properties of amnion for use as a scaffold in tissue engineering (11). The carbodiimide used was 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride. This approach to chemical crosslinking of proteins has the advantage that only non-toxic, water-soluble urea derivatives are side products. In addition, the amino acid side chains involved in the crosslink differ from those that react with glutaraldehyde. The carbodiimide agent facilitates amide bond formation between amino groups (lysine, amino end of protein chains) on one protein chain and carboxyl groups (aspartate, glutamate, carboxyl end of protein chains) on another chain.

Amniotic membrane (0.9-1.3 mg) was treated with varying concentrations of carbodiimide (0 to 20 mM) and 0-5 mM NHS (N-hydroxsuccinimide) in 0.05 M MES (N-morpholino)-ethansulfonic acid) buffer for 24 h at room temperature following the procedure of Ma et al. (11). The AM was thoroughly washed in distilled water to remove excess reagents and byproducts. The membrane was then cut and incubated with collagenase for 20 hr as described above. The free amine present in the supernatant was analyzed and the percent inhibition of AM degradation calculated relative to untreated control AM. An example of the results is shown in Figure 7. Total inhibition of degradation was achieved using 20 mM carbodiimide and 80% inhibition of degradation was observed using 10 mM. Similar results were found in three experiments. This result was substantiated by analysis of the rate of collagenase digestion of carbodiimide-treated and untreated amnion as shown in Figure 7B. ***These results indicate that a non-toxic carbodiide derivative completely inhibits collagenase-induced degradation of AM.***

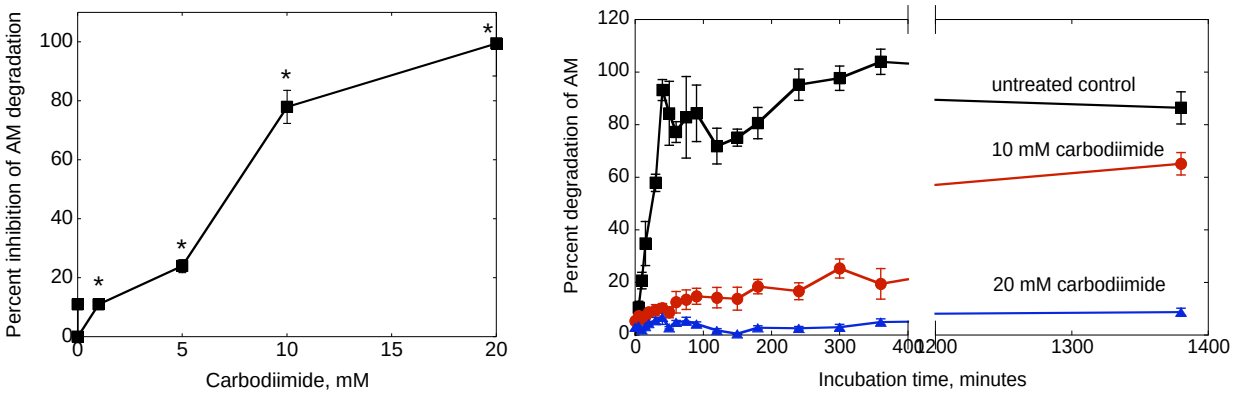

Figure 7. Crosslinking of amniotic membrane with carbodiimide. Percent inhibition of collagenase digestion of AM by treatment with varying concentrations of carbodiimide for 24 hr. * $p < 0.05$ compared to untreated control AM.

1.a.5. Summary

Table 1 summarizes the levels of protection against enzymatic degradation that were produced by treating AM with several crosslinking methods that operate by different mechanisms (table on next page).

Table 1. Summary of results from testing the ability of protein crosslinking agents to protect amniotic membrane against enzymatic degradation.

Crosslinking treatment	Treatment conditions	Number of trials	Maximum percent inhibition of degradation relative to untreated control [a]
Control, no treatment	----	25	---
RF-5P, 1%	50-200 J/cm^2	10	35 ± 12 at 100 J/cm^2
RB, 0.1%	2.5-20 J/cm^2	10	55 ± 13 at 10 J/cm^2
UVC	15-120 J/cm^2	4	43 ± 6 at 120 J/cm^2
Glutaraldehyde	10-50 μM, 30 min	4	100 ± 3 at 50 μM
Carbodiimide	1-20 mM, 24 hr	3	100 ± 5 at 20 mM

[a] Samples were incubated with 1% collagenase at 37°C for 20 hr.

Specific aim 1.c. <u>Assess biochemical and structural alterations in cornea of rabbit model for ectropion.</u> Create ectropion by blepharoplasty in New Zealand white rabbits. Assess epithelial defects and corneal ulcers. Measure inflammatory cytokines and proteolytic enzymes.

The animal study protocol was approved on April 16, 2010 by the USAMRMC Animal Care and Use Review Office (ACURO) for the use of rabbits and was approved by the Brooke Army Medical Center IACUC ((IACUC Protocol Number A-2009-02). The animal model for ectropion (rabbits) will be created when the USA ISR vivarium and animal surgery facilities are completed (anticipated in August, 2010). The laser and other equipment have been ordered, technical staff is being hired and arrangements are being made to obtain a commercial supply of amnionic membrane. A more complete description of the activities at Brooke Army Medical Center is included in the Annual Report for the partnering Grant Agreement, W81XWH-09-2-0069, Col Anthony J. Johnson, MD, PI.

Specific aim 1.d. Determine whether the relative degradation rates of modified amnion in vivo correlate with those measured in vitro. Amnion with differing degrees of protein crosslinking will be secured to rabbit cornea. The loss of amnion will be followed by slit lamp examination and histology.

As described above for Specific aim 1.c, these animal studies will be initiated when the USA ISR vivarium and animal surgery facilities are completed (anticipated in August, 2010).

KEY RESEARCH ACCOMPLISHMENTS:

- Developed a fluorescence-based assay to measure enzymatic degradation of modified amniotic membrane that can be used for small size samples (1 mg) and which efficiently tests large numbers of samples. This assay made it possible to optimize experimental conditions and evaluate multiple crosslinking agents.

- Identified the conditions for treating amniotic membrane with five protein crosslinking agents to provide maximum protection against enzymatic degradation. The five agents operate by different molecular mechanisms, thus providing different crosslinks that may show different effectiveness for inflamed eyes in vivo.

- The relative levels of maximum protection against enzymatic degradation of amniotic membrane were found to be: Glutaraldehyde = carbodiimide > Rose Bengal/green light > UVC radiation > riboflavin-5-phosphate/blue light

REPORTABLE OUTCOMES: None during this reporting period.

CONCLUSION:

In this reporting period, we completed the initial phase of our project with the goal of developing methods to protect the eyes of patients with severe facial burns and scarring. These patients cannot blink normally or close their eyes, leading to damage to the ocular surface and possibly the need for corneal transplant. Our approach is to stabilize amniotic membrane patches against degradation by the proteolytic enzymes present in inflamed eyes of these patients.

We determined the levels of protection afforded by five methods (including light-initiated methods) to crosslink proteins (largely collagen) in amniotic membrane against degradation by collagenase in an in vitro test system. The crosslinking methods differed in their molecular mechanisms for protein crosslinking, thus providing fundamental information as well as useful information for developing protective amnion patches. In addition, these different mechanisms may provide the ability to crosslink the amnion proteins while preserving the pro-healing properties present in the membrane. All of the methods protected the amnion patches in an in vitro assay system; their levels of effectiveness varied from 35 to 100%. These results are important for our goal because they provide the basis for selecting the crosslinked amnion patches to be tested in an in vivo model of eye inflammation and for testing the effect of protein crosslinking on the pro-healing factors present in the membrane. We are now ready to proceed to these next steps in developing protective patches for preserving ocular health in patients with severe facial burns.

REFERENCES:

1. Bantan-Polak T, Kassai M, Grant KB. A comparison of fluorescamine and naphthalene-2,3-dicarboxaldehyde fluorogenic reagents for microplate-based detection of amino acids. Anal Biochem. 2001 Oct 15;297(2):128-36.
2. Wollensak G, Spoerl E, Seiler T. Riboflavin/ultraviolet-a-induced collagen crosslinking for the treatment of keratoconus. American journal of ophthalmology. 2003 May;135(5):620-7.
3. McCall AS, Kraft S, Edelhauser HF, Kidder GW, Lundquist RR, Bradshaw HE, et al. Mechanisms of corneal tissue cross-linking in response to treatment with topical riboflavin and long-wavelength ultraviolet radiation (UVA). Investigative ophthalmology & visual science. 2010 Jan;51(1):129-38.
4. Verweij H, Dubbelman TM, Van Steveninck J. Photodynamic protein cross-linking. Biochimica et biophysica acta. 1981 Sep 21;647(1):87-94.
5. Ibusuki S, Halbesma GJ, Randolph MA, Redmond RW, Kochevar IE, Gill TJ. Photochemically cross-linked collagen gels as three-dimensional scaffolds for tissue engineering. Tissue engineering. 2007 Aug;13(8):1995-2001.
6. Mulroy L, Kim J, Wu I, Scharper P, Melki SA, Azar DT, et al. Photochemical keratodesmos for repair of lamellar corneal incisions. Investigative ophthalmology & visual science. 2000 Oct;41(11):3335-40.
7. Yao M, Yaroslavsky A, Henry FP, Redmond RW, Kochevar IE. Phototoxicity is not associated with photochemical tissue bonding of skin. Lasers in surgery and medicine. 2010 Feb;42(2):123-31.
8. Lambert CR, Kochevar IE. Electron transfer quenching of the rose bengal triplet state. Photochemistry and photobiology. 1997 Jul;66(1):15-25.
9. Fujisato T, Tomihata K, Tabata Y, Iwamoto Y, Burczak K, Ikada Y. Cross-linking of amniotic membranes. J Biomater Sci Polym Ed. 1999;10(11):1171-81.
10. Jayakrishnan A, Jameela SR. Glutaraldehyde as a fixative in bioprostheses and drug delivery matrices. Biomaterials. 1996 Mar;17(5):471-84.
11. Ma DH, Lai JY, Cheng HY, Tsai CC, Yeh LK. Carbodiimide cross-linked amniotic membranes for cultivation of limbal epithelial cells. Biomaterials. 2010 Sep;31(25):6647-58.